Molecular cloning and analysis of the Catsper1 gene promoter

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ABSTRACT: CatSper channels are essential for hyperactivity of sperm flagellum, progesterone-mediated chemotaxis and oocyte fertilization. Catsper genes are exclusively expressed in the testis during spermatogenesis, but the function and regulation of the corresponding promoter regions are unknown. Here, we report the cloning and characterization of the promoter regions in the human and murine Catsper genes. These promoter regions were identified and isolated from genomic DNA, and transcriptional activities were tested in vitro after transcription into human embryonic kidney 293, mouse Sertoli cells 1 and GC-1spg cell lines as well as by injecting plasmids directly into mouse testes. Although the human and murine Catsper promoters lacked a TATA box, a well-conserved CRE site was identified. Both sequences may be considered as TATAless promoters because their transcriptional activity was not affected after deletion of TATA box-like sites. Several transcription initiation sites were revealed by RNA ligase-mediated rapid amplification of the cDNA 5’-ends. We also found that the immediate upstream region and the first exon in the human CATSPER1 gene negatively regulate transcriptional activity. In the murine Catsper promoter, binding sites for transcription factors SRY, SOX9 and CREB were protected by the presence of nuclear testis proteins in DNase degradation assays. Likewise, the mouse Catsper promoter exhibited transcriptional activity in both orientations and displayed significant expression levels in mouse testis in vivo, whereas the suppression of transcription signals in the promoter resulted in low expression levels. This study, thus, represents the first identification of the transcriptional control regions in the genes encoding the human and murine CatSper channels.

Key words: CatSper channel / gene regulation / transcription / promoter / testis

Introduction

The cation sperm channel (CatSper) has been characterized as a Ca2+-permeable transmembrane protein associated with hyperactivation, chemotaxis and the acrosomal reaction. The human and murine Catsper genes are expressed specifically in sperm, and both proteins are located in the principal piece of the flagellum (Ren et al., 2001; Li et al., 2006). Catsper was the first of four identified genes encoding for proteins similar to the pore-forming α1 subunit of voltage-gated Ca2+-channels (Ren et al., 2001). Unlike other CatSper proteins, Catsper1 contains a histidine-rich region that allows channel activation with slight changes in intracellular pH (Kirichok et al., 2006; Lishko and Kirichok, 2010). Catsper channels also include auxiliary subunits called Catsper-β, Catsper-γ and Catsper-δ (Liu et al., 2007; Wang et al., 2009; Chung et al., 2011).

CatSper channels have been associated also with non-genomic actions of progesterone in human sperm, although progesterone seems not to affect murine CatSper channels (Lishko et al., 2011; Strunkker et al., 2011). Likewise, CatSper channels seem to mediate Ca2+-influx to the flagellum that induces increased Ca2+ levels in the sperm head by releasing Ca2+ from the internal stores and contributes to the Ca2+-dependent acrosomal reaction (Xia and Ren, 2009; Olson et al., 2010). Interestingly, disruption of the murine Catsper1 gene yields homozygous male offspring that fail to engender pregnancies as...
a result of sperm abnormal motility. Hyperactivated motility is necessary for exiting the reservoir in the oviductal epithelium and penetrating the oocyte ‘zona pellucida’ (Carlson et al., 2003; Ho et al., 2009).

CatSper1 transcripts have been detected in the testis of mice, human, and pigs, as well as in ejaculated human sperm. Murine CatSper genes are expressed from post-natal day 18 to the adult testis stage, when the maximum levels of expression are found (Schultz et al., 2003; Nikpoor et al., 2004; Li et al., 2007). The expression profiles of the four CatSper transcripts during post-natal development are distinct, which suggests independent transcriptional regulation (Li et al., 2007). Interestingly, the regulation of CatSper1 expression directly correlates with the onset of spermatogenesis and sexual maturation (Nikpoor et al., 2004; Li et al., 2006; Song et al., 2011).

CatSper1 channel expression seems to be also essential for normal human male fertility. Human CATSPER1 gene alterations have been associated with autosomal-recessive male infertility, and sequence analysis has revealed insertion mutations that produce frameshifts resulting in the presence of a premature stop codon within the first exon (Avenarius et al., 2009). Likewise, in some cases of male infertility, a reduction in CATSPER1 transcript levels and diminished sperm motility have been observed (Nikpoor et al., 2004). In spite of this, studies regarding transcriptional regulation of the CatSper1 gene have not been previously undertaken. In this report, we describe the first analysis and characterization of the mouse and human CatSper1 gene promoters.

Materials and Methods

Reporters constructs

DNA was isolated from a BAC clone (DH10B pBAC RCPI8753F22692QC) obtained from the Roswell Park Institute Library. A ~1700-bp region of the first exon and a region of ~2000 bp upstream from the human CATSPER1 gene were obtained using a double enzymatic digestion (Xhol and HindIII) and ligated into Sall and HindIII sites within the Renilla luciferase reporter vector pRL-null (Promega, Madison, WI, USA). Other constructs were obtained from the Roswell Park Institute Library. A DNA was isolated from a BAC clone (DH10B pBAC RPCIB753F22692QC) for the ES_hrGFP_1a vector that encodes green fluorescent protein (GFP) as the reporter. The CatSper1 promoter was placed in substitution of the cytomegalovirus (CMV) promoter in the pRES_hrGFP_1a vector to generate the p798CatS-GFP vector. An Nsil-BamHI fragment obtained from the pCRTOPO-798CatS vector deleted a fragment (nt -66 to +23) at the 3’ end. This fragment was cloned into the Nsil-BamHI sites of the pRES_hrGFP_1a vector without the CMV promoter to obtain the pbasalCatS-GFP construct.

The integrity and orientation of the cloned sequences were confirmed by automatic DNA sequencing using an ABI PRISM 310 sequence analyzer (Perkin-Elmer Applied Biosystems) with the forward (5’GCTCACA TGGCTCGAC3’) and reverse primers (5’CCAGTGCTCAGGACAC3’) from the pRL-null, RVP primer3-binding site of the pGL3basic vector and reverse primer (5’CCTTGAGTGGCTGAGTC3’) from pRES.

Promoter analysis

The promoter nucleotide sequence was cloned, and its identity was confirmed using the BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi) that identified similarities with the 5’-flanking region of the human CATSPER1 gene (ID:117144). The cloned nucleotide sequence and the CatSper1 promoter regions obtained from the human, rat and mouse sequences were aligned using the ClustalX program (http://www-igbmc.u-strasbg.fr/BioInfo/) (Thompson et al., 1997). Promoter prediction was performed using EIDorado/Gen2Promoter (http://www.genomax.de). The prediction of putative transcription binding sites within the CatSper1 promoters was performed using TFSEARCH (www.cbr.cmp.uct/research/dbs/TFSEARCH.html), Mat-inspector (http://www.genomatix.de) and Mapper programs (http://bio.chip.org/mapper).

RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE)

Total RNA from human testis was purchased from Clontech (Palo Alto, CA, USA). RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) reactions were performed using a First-Choice kit (Ambion, TX, USA) according to the manufacturer’s instructions. Briefly, 2 μg of total RNA was treated with calf intestine alkaline phosphatase at 37°C for 1 h and removed using one volume of phenol:chloroform. Precipitation was performed with 150 μl of isopropanol on ice for 10 min. The RNA pellet was rinsed with cold 70% ethanol, resuspended in nuclease-free water and treated with tobacco acid pyrophosphatase at 37°C for 1 h, and next the RNA was ligated to the 5’RACE adapter (5’-GCUGAUUGGCGAUAAGAAUCGUCCGUUUUGCUGGUUGAUGAAA-A). For the reverse transcription reaction, 1 μl of the RNA ligated reaction and 1 μl of random decamers were used, and the reaction mix was incubated at 42°C for 1 h.

The first PCR step for the outer 5’RML-RACE was performed using 1 μl of RT reaction, 10 pM of the 5’RACE outer primer (5’-GTGGAGACTGCTGGTGGC-3’) and 10 pM of the CATI outer primer (5’-GTGGAGACCTGGCTGGTGGC-3’). The reaction conditions were as follows: 94°C for 3 min, 35 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) and an extension step of 7 min at 72°C. The nested PCR for the inner 5’RML-RACE was performed using 1 μl of the initial PCR amplification reaction, 10 pM of the 5’RACE inner primer (5’-CGCCGATCGGCCAACACTGCTGGTGGTGGC-3’) and 10 pM of the CATI inner primer (5’-CCTCATCTGGACCTGCTGGTGGC-3’). The cycling conditions for the reaction were similar to those applied for the first PCR. The products from the nested PCR were separated by agarose gel electrophoresis, cloned into the pJET 2.1/Blunt vector (Fermentas, Burlington, Canada) and detected via PCR screening of the bacterial colonies. Lastly, the transcription start site (TSS) of the human CATSPER1 gene was verified via automatic sequencing.
Cell culture, transfection and luciferase assays

The human embryonic kidney cell line (HEK293), mouse Sertoli cells (MSC1) and spermatogonial germ cells (GC-1spg) were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% inactivated fetal bovine serum (GIBCO/BRL Life Technologies, Grand Island, NY, USA) and 1% HEPES. Cells lines were cultured in the presence of 50 μg of penicillin, 50 μg of streptomycin and 100 μg of neomycin per millilitre of media (GIBCO) and maintained in a humidified incubator at 37 °C and 5% CO₂.

Twenty-four hours before transfection, 2.5 × 10⁵ cells/well of each cell line were plated in 24-well plates. The plasmid DNAs were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. To normalize for transfection efficiency, cells were co-transfected with the Photinus luciferase expression plasmid, pGL3-CMV for pCAT constructs or with Renilla luciferase expression plasmid pRL-CMV for pCatS constructs (Promega). Next, 500 ng of the reporter construct and 20 ng of the control vector were applied to each well. The medium was replaced 24 h post-transfection, and the cells were lysed and subjected to luciferase activity assays using the Dual-Luciferase Reporter Assay System (Promega) 48 h later. The pGL3-CMV or pRL-CMV vectors were used as positive controls according to the construct used. Three independent transfections were performed in triplicate.

Nuclear protein extraction from cell lines and testis

Confluent MSC1, HEK293 and GC-1spg cells were harvested and testes dissected from CD1 male adult mice. Nuclear extracts from these preparations were isolated with a ProteoJetTM Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) according to the supplier’s instructions for cultured cells or tissue samples. Nuclear protein concentration was determined via Bradford assay.

DNase protection assays

A 798-bp amplicon of the CatSper1 promoter was obtained by PCR from the pCatS798 vector and labelled with 10 U/μl of T4 polynucleotide kinase in the presence of 10 μCi of α³²P-dATP; the nucleotides were then removed by centrifugation in a Centrffi-Sep column (Princeton Separations Inc., Princeton, NJ, USA). Next, the amplicon was used for electrophoretic mobility shift (EMSA) and DNase protection (footprinting) assays. Binding of different concentrations of testis nuclear proteins to the CatSper1 promoter probe was performed in binding buffer (10 mM HEPES pH 7.9, 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 1 μg poly dA-dT and 10% glycerol) with 20 ng, 0.4 and 2 μg of nuclear proteins in a final volume of 50 μl for 20 min on ice. DNA protein complex formation was confirmed using non-denaturizing gels that were dried and then exposed to film for 2 h and revealed.

For footprinting assays, 3 μg of the 798-bp CatSper1 promoter PCR product and 10 μg of nuclear proteins from murine testis or cell lines were incubated at room temperature in binding buffer (20 mM HEPES (pH 7.9), 50 mM KCl, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1.0 mM PMSF, 0.05% Nonidet P-40) in a total volume of 50 μl. After 20 min, 50 μl of a 2X cofactor solution (50 μl of 10 mM MgCl₂ and 5 mM CaCl₂) and 0.01 U of DNase were added. Cleavage was terminated by the addition of 200 μl of stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8 and 40 μg tRNA). The samples were extracted with phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in 4 μl of loading buffer (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA and 95% formamide). The DNase I products were separated in a denaturing 6% polyacrylamide gel (7 M Urea, 1x TBE, 40 ul tetramethylthelyenediamine and 10% ammonium persulphate) and detected using the DNA silver-staining system (Promega). Maxam–Gilbert sequencing reactions of pCatS798 with CatS1rev primer were run to locate sequence positions of protected regions.

In vivo transfection

CD1 male 8-week-old mice were maintained under constant temperature (28 °C) and a 12-h light/12-h dark cycle with free access to pelleted food and water ad libitum in the animal facility at the Instituto Nacional de Ciencias Médicas y Nutrición ‘Salvador Zubirán’ (INCMNSZ). The Animal Resources Laboratory, the animal care procedures and the programs at the INCMNSZ were fully accredited by the Office of Laboratory Animal Welfare of the US Public Health Services under the animal welfare assurance # A5600-1.

Three independent experiments were performed with four male mice. Animals were anaesthetized with ketamine-xylazine (22 and 1.1 mg/kg, respectively) via i.m. injection. Once the mice were anaesthetized, the right testis was washed with 60% ethanol, and the fur was removed using a scalpel and a 32G ultrathin needle. The injection was applied until the trypan blue was visible in 80% of the seminiferous tubules.

For each animal, the right testis was treated as the experimental organ, and the left testis remained untreated and was considered as a control. After DNA injection, the mice were treated with antibiotics (Ampicillin, 0.1 mg/kg/i.m.) and pain medication (Ketoprofen, 3 mg/kg/i.m.); one dose was administered daily over 3 days. These treatments failed to cause noticeable damage to the testes, and histopathological abnormalities were not observed. The animals were maintained for 1 week before they were euthanized, and their testes were then prepared for histological analysis using epifluorescence microscopy (Olympus BX51).

Results

Analysis of the human and murine Catsper1 promoters

Two promoters for the human CATSPER1 gene were detected. The first one of 739-bp overlapped the first exon (Fig. 1) and the other of 601 bp was located ~17.5 kb upstream of the first exon. A bioinformatic analysis revealed that the Catsper1 genes of several species, including Bos taurus, Macaca mulatta and Pan troglodytes also have two promoters (Fig. 2A). In P. troglodytes, the gene comprises 18 predicted exons with the 5’ end extending ~17 kb in which the first exon is located next to the distal promoter, whereas in humans, the first exon of the CATSPER1 gene overlaps with the proximal promoter, and the distal promoter may be considered as remnant of a gene that has been reduced in length. In contrast, only one proximal promoter could be predicted for the Mus musculus and Canis familiaris Catsper1 gene.

The 739-bp human promoter proximal sequence contains the start codon and the first 33 codons encoding the amino terminal of the CatSper1 protein and SRY, CRE and CAAT-box sites (Fig. 1A).
When aligned with mouse and rat sequences, all promoters showed a homology of $\approx 67\%$ and a differential distribution of the binding sites for transcriptional factors with the exception of one CRE site and one CAAT box (Fig. 2B). Interestingly, although a TATA box close to the TSS was observed in the rat promoter, this sequence was not present in the human and the murine promoters. This alignment revealed also a well-conserved CRE site next to the TSS and the presence of a CAAT box upstream of the TATA box in the rat promoter.

**Mapping of the TSS**

We next sought to determine whether the TSS in the human gene was located within the promoter region (nt $-500$ to $+236$) using 5’ RLM-RACE. The first extension with the outer primers should yield a PCR1 product of 306 bp. For the nested PCR with inner primers, a 214-bp PCR2 product was predicted (Fig. 1B). Fifteen candidates with cloned inserts were analysed; 3 candidates displayed a long insert of $\approx 350$ bp, 4 candidates displayed a $\approx 300$-bp insert and 9 candidates displayed $\approx 250$-bp amplicons. All these clones were verified by automatic sequencing. The insert lengths and their corresponding 5’ ends were A174, T135 and A130 bp. Hence, the TSSs in the CATSPER1 promoter sequences were located at positions $-213$, $-198$ and $-193$ from the start codon. Although we observed a difference of three nucleotides between the NCBI (NM-053054.3)-assigned TSS (adenine at position $-138$) and our experimental TSS

**Figure 1** Nucleotide sequence of the human CATSPER1 promoter and localization of the TSS. (A) A sequence of 740 bp (430 bp in the flanking 5’-upstream region and 300 bp in the first exon) of the human CATSPER1 gene is depicted (ID:117144). Transcription factor binding sites for SRY, Oct1, YY1, SFI GCNF, ER, CRE, Sp1, CAAT-BF and GATA1 are underlined. The putative signals for the basal transcription machinery are represented by bold letters. (B) PCR strategy for 5’RACE showing the Catsper1-specific primers and 5’RACE primers for the 5’ ligated RNA adapter to testis RNAm (black arrows). Expected PCR products are marked as PCR1 and PCR2. The TSSs examined in this study are marked as single bold letters, the start codon is indicated by three bold letters and template sequences for oligonucleotides used for 5’RACE-PCR are located above the arrows.
(adenine at position −135), we found that this base was well conserved among all the promoters analysed, except in *M. musculus*, where it was substituted by a G (Fig. 2B). These experimental TSSs were present in the *CATSPER1* promoter region of all our constructs.

### Analysis of transcriptional activity in transfected cell lines

To identify the regions that regulate the transcriptional activity of the human *CATSPER1* promoter, a region with the first exon and +2153 bp upstream from the TSS was cloned into a promoterless luciferase pRL-null vector (Fig. 3). The pCAT1 construct and the corresponding derivatives were transfected into the spermatogenic mouse cell line GC-1spg, and Renilla luciferase reporter activity was monitored. As can be seen in Fig. 4A, a 3-fold induction was observed with the pCAT739 construct (a proximal promoter of 739 bp) that spanned from nt −629 to +102 with respect to the long construct pCAT1 that displayed low transcriptional activity. Interestingly, the loss of the first exon (pCATD3′ construct) resulted in only ≏2-fold increase in activity, suggesting that the first exon has downstream negative regulatory elements for transcription. A similar effect was observed when another exonic deletion (pCATDSE construct; nt −2115 to +1153) was used (Fig. 4A).

We next compared transcriptional activities among different cell lines. To this end, we reported the *CATSPER1* promoter transcriptional activities as a percentage relative to the transcriptional activity of the CMV promoter. In the MSC1 cells, the transcriptional activity was lower than in GC-1spg cells and was similar with different constructs, suggesting a differential transcriptional control in this cell line (Fig. 4B). Likewise, the transcriptional activity was also low when the constructs

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**Figure 2**  
In silico analysis of the *Catsper1* promoters. (A) Promoter prediction over 30 kb of the genomic DNA upstream of the *Catsper1* gene. Two types of promoter (yellow bars), a distal promoter and a proximal promoter were found adjacent to the *Catsper1* genes; the genomic exons are represented with green bars. (B) Alignment of the sequences of ≏280 bp for the *Catsper1* gene promoter region in several species as indicated. The binding sites for various transcriptional factors and the start codon (−134) are indicated within red boxes. The experimentally determined TSSs located at positions +1, +37 and +42 are also indicated in red rectangles. A SNP was identified at nucleotide +430 that varied between the cloned promoter and the GenBank *CATSPER1* sequence (ID:117144).
were expressed in HEK293 cells (Fig. 4C). Lastly, the activity of the proximal promoter (pCAT739 construct) displayed a high activity in GC-1spg cells in comparison with Sertoli cells and the HEK293 cells (Fig. 4D).

CAAT and TATA boxes may not contribute to basal transcription

The *in silico* analysis of the CATSPER1 promoter revealed one TATA-like box sequence near the TSS, two CAAT boxes and a GC box upstream of the TSS (Fig. 1A). To test whether the CAAT boxes and the TATA-like box contribute to the recruitment of the basal transcriptional machinery, a construct was designed, where these regions were deleted (pCATΔbasal), and its reporter activity was monitored in HEK293 and MSC1 cells. As shown in Fig. 5A and C, similar levels of activity between the human pCAT739 and pCATΔbasal constructs were observed in both cell lines. Similar results were observed after the elimination of the TATA-like boxes in the CatSper1 murine promoter (Fig. 5B and D). Together, these data rule out a contribution of the CAAT and TATA-like sequences to basal transcription of the CATSPER1 gene. Interestingly, *in silico* analysis showed that other downstream sites such as Sp1, SRY and Gata1 (Fig. 1A) can be found in the CATSPER1 promoter, as well as a conserved CRE-binding site located next to the TSS that could function as a substitute for the CAAT and TATA sequences in transcription.

The murine CatSper1 promoter has bidirectional transcriptional activity

To assess whether a similar size promoter region of the murine CatSper1 could display transcriptional activity, a ~800-bp region (nt −775 to +23 from the start codon) was cloned in sense orientation adjacent to a Photinus luciferase reporter in the promoterless pGL3-basic vector (pCatS798 construct). The same CatSper1 promoter regions were cloned in antisense orientation into phLuc to create the pCatS798AS and pCatS1215AS constructs. The p798CatS-GFP plasmid contained the murine CatSper1 promoter (798 bp), and the pΔbasalCatS-GFP plasmid contained the same region with a deletion between nucleotides −66 and +23 to the TSS; both preceded the GFP gene.
Testis nuclear proteins bind to the *Catsper1* promoter at transcriptional factor sites

The binding of testis nuclear factors to the *Catsper1* promoter was tested via EMSA and footprinting. Nuclear protein extracts were obtained from mouse testis, MSC1, HEK293 and GC-1spg cells. Binding assays with nuclear proteins isolated from mouse testis were performed to determine whether 2 μg of nuclear protein was sufficient to retain 100% of the γ32P-labelled probe. The results indicated that binding with 20 and 400 ng of nuclear proteins can be displaced by an excess of 100 ng of an unlabelled promoter fragment in competition assays (Fig. 6A and B).

Next, a concentration of DNAse1 of 0.01 U was used in a protection assay to obtain a controlled digestion fragment from the *Catsper1* promoter. Protection from DNAse1 degradation was observed in several regions of the *Catsper1* promoter after incubation with nuclear proteins from murine testis, MSC1, HEK293 and GC-1spg cells (Fig. 6C). The binding sequences for the transcription factors Sry, CAATbox (or Sox9 overlapped), TATA-like box and CRE/GNCF next to the TSS were located in the protected regions according to the coordinates of the *Catsper1* promoter given in Fig. 2B.

The murine *Catsper1* promoter directs the expression of GFP in mouse testis

We next tested the activity of the *Catsper1* promoter region in the testicular environment. To this end, a 798-bp *Catsper1* promoter sequence followed by the GFP was constructed as a reporter protein for in vivo expression. The pCatS798-GFP plasmid harbours the complete sequence −775 to +23 of the *Catsper1* promoter that was inserted in substitution of the CMV promoter in the pRES_hrGFP_1a vector to direct GFP expression. A second pΔbasalCatS-GFP construction devoid of the −66 to +23 region of the *Catsper1* promoter (Fig. 3), which contains the identified signals for basal transcription and the annotated TSS for *Catsper1* expression (accession # NM_139301.2), and the pRES_hrGFP_1a vector with a CMV promoter for the transcription of GFP were used as controls.

The constructs were inoculated (20 μg of each plasmid) in CD1 adult mice via puncture of the right testicle. Histological sections were obtained 7 days after inoculation. The use of the truncated construct (pΔbasalCatS-GFP) resulted in low GFP expression, indicating low transcriptional activity (Fig. 7A). In contrast, the use of the construct containing the intact promoter (p798CatS-GFP) resulted in strong fluorescence signals in germ cells within the seminiferous tubules (Fig. 7B) indicative of strong expression of GFP driven by the *Catsper1* promoter that exceeded the expression levels observed with the control pRES_hrGFP_1a vector (Fig. 7C), in which GFP transcription is driven by the CMV promoter. These data also suggest that
the cloned Catsper1 promoter region is functional in the testicular environment, as the gene expression occurs in germ cells towards the centre of the seminiferous tubules.

**Discussion**

Studies using knockout mice and mutations of the Catsper1 gene have demonstrated the importance of this channel in male germ fertility. Here, we show experimental evidence for the characterization of transcriptional control regions in the genes encoding the human and murine CatSper channels.

Once the human promoter was isolated, we started its characterization by identifying the TSS. The human Catsper1 mRNA sequence reported in the NCBI (GenBank: NM_053054) includes an untranslated region of 138 nucleotides at the 5′ end, in which the annotated TSS is an adenine. Here, we experimentally identified a TSS only three nucleotides downstream of the annotated TSS at a well-conserved adenine within an Inr sequence (cgAgaat) that had a better identity with the consensus Inr sequence YYANWYY than the previous annotated TSS. Additionally, in the present work, other downstream TSSs were also identified near to the 5′ end of the cDNA clone (BC036522.2). Consistent with this, multiple TSSs are frequently found in TATAless promoters. In addition, it is well known that TSSs changes in testis-specific promoters may occur in response to distinct inductor signals (Hagiwara et al., 1996; Zheng and Martin-Deleon, 1999; Hata and Ohtsuka, 2000; Gaviraghi et al., 2008).

We found that the human CATSPER1 gene transcription is directed by a proximal promoter that included a 243-bp portion of the first exon. Therefore, the promoter sequence cloned here includes the first exon that includes several transcription factor binding sites. In this study, we also describe that the first exon exerts a negative cis effect on transcription. In contrast, the 5′ upstream cloned region (2153-bp) did not display inhibitory effects on the transcriptional activity of the CATSPER1 promoter, most likely because this region contains multiple binding sites for transcriptional factors such as SRY, OCT1, SF1, GCNF, CRE, CAATbox, Sp1, GATA1 and YY1 that generally exert a positive effect on transcription. However, confirmation of this hypothesis requires further study. Likewise, a Sp1 binding site is located downstream of the TSS in the CATSPER1 promoter (Fig. 1A). The Sp family of transcription factors binds to GC-box motifs in promoters and may play a key role in the transcriptional
HEK293 cells and with the observation that the sperm nucleus of MSC1 and HEK293 cells revealed some transcriptional activity, as pre-
moter must be specific for the germ-cell lineage, its transfection in levels and localization in the human germ cells suggest that the pro-
CATSPER1 (GC-1spg) are suitable for studying the transcription of the proximal sequences (Suske, 1999).

Activation of promoters that lack either TATA or CAAT consensus sequences (Suske, 1999).

Our findings in cell lines suggest that the spermatogonial cells (GC-1spg) are suitable for studying the transcription of the proximal human CATSPER1 promoter. However, although Cat sperl expression levels and localization in the human germ cells suggest that the pro-
moter must be specific for the germ-cell lineage, its transfection in MSC1 and HEK293 cells revealed some transcriptional activity, as previ-
ously characterized (Feng et al., 2000; Borchert et al., 2003; Gaviraghi et al., 2008). This is also consistent with previous studies showing that other germ cell-specific genes display transcriptional activity in HEK293 cells and with the observation that the sperm nucleus glutathione peroxidase (snGPx), expressed in the late spermatids, is also detected in testis, kidney and HEK293 cells (Borchert et al., 2003). It is also worth mentioning that the CATSPER1 promoter was recognized by the transcription machinery in the three cell lines tested, possibly because transcriptional factors that negatively regulate its expression in somatic cells are not present in these cell lines.

Concerning the murine promoter, it was interesting to observe transcriptional activity in the antisense orientation, suggesting that the Catsperl promoter may act as a bidirectional promoter. Indeed, the transcriptional activity of the antisense promoter was stronger than the sense Catsperl promoter, suggesting that both promoters compete for the transcriptional machinery in a way that is disadvanta-
geous for the sense Catsperl promoter. Remarkably, the murine Catsperl gene (located on chromosome 19) displays a long non-
annotated upstream region spanning 10 488 bp up to the GM7074 gene (RNA polymerase II-associated protein 2 pseudogene) and 27 002 bp to the galactose-3-O-sulphotransferase 3 (Gal3st3) gene. These neighbour genes are transcribed in the same direction as the Catsperl gene. In contrast, the CST6 gene (cystatin E/M) located downstream of the 3’ end of the Catsperl gene is transcribed in the opposite direction. Furthermore, the human Gal3st3, CST6 and CATSPER1 genes kept the same arrangement in chromosome 11. The bidirectional transcriptional activity of the Catsperl promoter may produce either divergent gene expression or non-coding RNA expression.

Other testis-specific genes are also expressed by bidirectional pro-
moters. For example, the PURG gene located opposite to the WRN gene encodes the PURG-B protein (Liu and Johnson, 2002); the male-enhancer antigen-1 (Mea1) expressed in germ cells is transcribed from a testis-expressed bidirectional promoter that also drives the Peas gene (Ohinata et al., 2003); Haspin (Gsp2), a protein kinase expressed in haploid male germ cells is the product of a gene with a short bidirectional promoter of 193 bp shared by the Aed gene (Tokuhiro et al., 2007); and last, a repressor of the TATA box, the Dja gene (divergent from Ate1), results from the activation of the bidirectional Ate1 promoter that trans-
scribes a highly expressed isoform in the testis (Hu et al., 2006; Brower et al., 2010).

The putative signals for recruiting the transcriptional machinery within the Catsperl promoter, such as the CAAT and TATA-like boxes identified by in silico analysis, were also examined in this study. First, the deletion of these sequences in the promoter resulted in similar levels of activity in both the pCATΔbasal and pCAT739 vectors when compared with the promoter-less pRL-null vector. Although no consensus TATA box sequences were identified in the Catsperl gene promoter, it is possible that other factors may recruit the transcriptional machinery upstream of the TSS as seen in several other TATAless promoters. Many testis-specific promoters, described as TATAless such as those associated with the Bax inhibitor-1, PURG-A and Oxtc2b genes, present multiple TSSs and use the CRE or Sp1 ele-
ments for transcriptional initiation (Jean et al., 1999; Liu and Johnson, 2002; Somboonthum et al., 2005).

Other upstream signals might control also the transcriptional initi-
ation of the Catsperl promoters. Our bioinformatic analysis revealed binding sites for spermatogenesis-specific transcriptional factors that may activate or repress the promoter such as SRY, CREB and

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Figure 6 The murine Catsperl promoter recruits transcriptional factors. (A) The labelled Catsperl promoter amplicon (798 bp) was examined for binding to nuclear proteins from mouse testis. Two micrograms, 400 and 20 ng, of nuclear proteins were bound to the promoter and resolved on a 6% non-denaturating polyacrylamide gel. (B) The same labelled probe was bound to either 20 or 400 ng of nuclear proteins in the presence of 100 ng of a specific DNA competitor (+). (C) Restriction footprinting analysis of the Catsperl promoter with testis and cell line nuclear proteins. The Catsperl promoter amplicon 3 μg was incubated with 10 μg of nuclear pro-
teins from mouse testis (lane 1), MSC1 (lane 2), HEK293 (lane 3), GC-1spg cells (lane 4) or naked DNA (−). The protected regions are indicated by arrowheads or denoted as vertical text indicating the binding sequence CRE. Horizontal text indicates the TATA-like box, CAATbox/SOX9 and SRY, between nucleotides +45 and −150 of the proximal Catsperl promoter. The lanes designates A, C, G, T corresponding to a Maxam–Gilbert sequencing obtained with the pCatS789 and CatS1 reverse primer.
CREM, as well as YY1 and GCNF (Fig. 1A). The protection conferred by the testis nuclear proteins on the SRY, CAAT-box and CRE binding sites of the *Catsper1* promoter suggests that these transcriptional factors may assist the recruitment of the transcriptional machinery or act as transcriptional transactivators of the promoter in germ cells (Martianov et al., 2010). Likewise, we identified one CRE binding site that overlaps with a GCNF site for the repressor germ cell nuclear factor that is sufficient to direct cell type-specific expression *in vivo* (Rajkovic et al., 2010). The promoter exhibited strong transcriptional activity in the seminiferous tubules *in vivo*, suggesting that the *Catsper1* promoter is a specific promoter recognized by the transcriptional machinery in germ cells.

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**Authors’ roles**

N.O. and J.H.-S. designed the study and the interpreted data. D.C. and E.A.-C. performed the bioinformatic analysis. E.A.-C., M.M.-R. and A.H.-R. coordinated the experimental work. E.T.-C. contributed to the *in vivo* experimentation. N.O. and E.T.-C. both analysed and interpreted the data and drafted the manuscript. J.H.-S. and R.F.
critically revised the manuscript for substantial intellectual content. All authors have read and approved the submitted manuscript.

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**Conflict of interest**

None declared.

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